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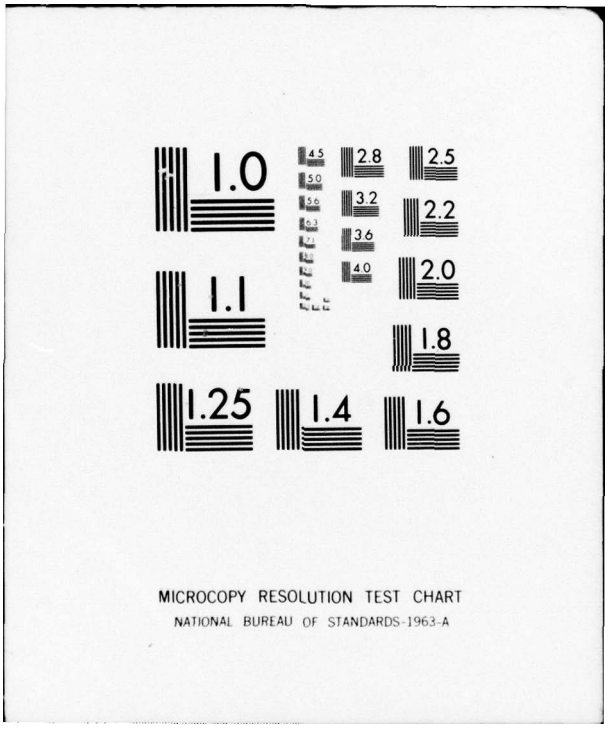
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The findings in this report are not to be construed as an official Department of the Army position, unless so designated by other authorized documents.

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Phagycytosis, macrophages, membrane transport, glucose and amino acid transport, lymphocytes, cell mediated immunity, staphylococcal infection, polymorphonuclear leukocytes, diphtheria toxin, Fragment A, pinocytosis, mouse L-cells, HEp-2 cells, athymic mice, activated macrophages

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The investigations completed concern the following areas of research: a) characterization of the glucose and amino acid transport systems of mouse and guinea pig macrophages and polymorphonuclear leukocytes. b) enhancement of glucose transport by macrophages following a phagocytic stimulus or immunologic activation and attempts to define the mechanism responsible for the increased uptake of the sugar. c) phagocytic and bactericidal activities of mouse peritoneal macrophages before and after activation by (over)

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immunological and non-immunological means. d) cell mediated immune phenomena associated with chronic staphylococcal infections of mice. e) uptake (pinocytosis) of diphtheria toxin and its fragment A moiety by non-phagocytic and phagocytic cells. f) interaction of diphtheria toxin with normal and transformed mammalian cells in culture, and g) natural resistance of athymic (T-cell deficient) mice to bacterial infections in the presence of activated macrophages in reticuloendothelial organs of athymic mice as factor in the anomalous resistance to infectious agents.

ENDOCYTOSIS AND TRANSPORT IN EUCARYOTIC CELLS

FINAL REPORT

PETER F. BONVENTRE

1 JUNE 1973 - 31 DECEMBER 1976

U. S. ARMY RESEARCH OFFICE

Contract/Grant Number: DA ARO D 31-124-73-G123
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UNIVERSITY OF CINCINNATI COLLEGE OF MEDICINE
CINCINNATI, OHIO 45267

1. FOREWORD

The primary objectives of the research activities covered by this grant were to study a number of biochemical and immunological properties of phagocytic cells derived from experimental animals. Particular attention was directed to membrane associated functions of the eukaryotic cells such as phagocytosis of particles, pinocytosis of proteins and carrier mediated transport of glucose and amino acids. The support provided by the grant has resulted in 8 published manuscripts, 2 currently in press, 2 submitted for publication and one other in preparation. Assuming that those submitted and the manuscript in preparations are published, 13 papers in all will have been published under the acknowledged auspices of the grant from the Army Research Office.

In addition to the support provided for the research activities leading to these publications, several graduate students have received M. S. or Ph.D. degrees, and several post-doctoral fellows have furthered their training under the auspices of the grant since its inception more than 3 years ago. A list of individuals associated with the work is appended to the technical report.

2. TABLE OF CONTENTS

N/A

3. LIST OF APPENDIXES

a) Graduate students and post doctoral fellows:

Allen Nickol, graduate student assistant, M. S.
Catherine B. Saelinger, Assistant Professor, Ph.D. (formerly post doctoral fellow)
David Straus, post doctoral fellow, Ph.D.
John Imhoff, research assistant, B. S.
Robert E. Baughn, graduate student assistant,Ph.D.
Antony Mukkada, Assistant Professor of Biological Sciences, Ph.D.
Casimir Woscinski, post doctoral fellow, Ph.D.

b) Reprints of papers published

Acquired cellular resistance following transfer of lymphocytes from mice infected repeatedly with Staphylococcus aureus. Robert E. Baughn and Peter F. Bonventre. Cellular Immunology 27: 287 (1976).

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Interaction of toxin of Corynebacterium diphtheriae with phagocytes from susceptible and resistant species. J. Infectious Diseases 131: 43, (1975).

Nonspecific resistance to Listeria monocytogenes in mice infected and elicited with Staphylococcus aureus. Robert E. Baughn and Peter F. Bonventre. Med. Microbiol. Immunol. 161: 243 (1975).

Phagocytosis and intracellular killing of Staphylococcus aureus by normal mouse peritoneal macrophages. Robert E. Baughn and Peter F. Bonventre. Infection and Immunity, 12: 346 (1975).

Cell-mediated immune phenomena induced by lymphokines from splenic lymphocytes of mice with chronic staphylococcal infection. Robert E. Baughn and Peter F. Bonventre. Infection and Immunity 11: 313 (1975).

Augmentation of glucose transport in macrophages after particle ingestion. Peter F. Bonventre and Antony J. Mukkada. Infection and Immunity. 10: 1391 (1974).

Membrane transport by mouse and guinea pig macrophages: characteristics of the glucose transport system. J. Reticuloendothelial Society 17: 20, (1975).

4. BODY OF REPORT

The diversity of research activities conducted make it mandatory that the report be divided in a number of brief summaries describing the pertinent data and conclusions drawn from them. The main thrust of the research was to investigate the functional and biochemical attributes of a variety of phagocytic cells from experimental animals with an aim to understanding of activities associated with the plasma membrane and internal membrane bound structures (i.e., pinocytic vesicles and phagocytic vacuoles). Some of the research was conducted in intact animals but the majority of experiments were done utilizing isolated phagocytic cells cultured in vitro. The following brief descriptions represent the research accomplished during the tenure of the grant between June 1, 1973 and December 31, 1976.

a) Membrane transport of glucose transport by macrophages in vitro culture. Glucose transport in guinea pig and mouse peritoneal macrophages was characterized. It was established that phagocytes from both animal species accumulate the sugar by facilitated diffusion. The transport system in both cases is saturable with a K_m of 0.7 mM and 1.2 mM for guinea pig and mouse macrophages, respectively. Macrophages from the unstimulated peritoneum accumulate glucose minimally but transport is enhanced significantly after 24 hr or more of in vitro maturation. Unaltered 2-deoxy-D-glucose is present in the intracellular sugar pool to the extent of 25 to 35% of the total after uptake, with the remainder being phosphorylated by cellular kinases. Since free sugar reflects the transport system per se, it was calculated that the intracellular level achieved was approximately 1.0 mM. Normal, dialyzed serum did not modify glucose uptake but Cytochalasin B abolished uptake completely.

b) Increase in glucose transport after phagocytosis by peritoneal macrophages. Guinea pig and mouse peritoneal macrophages in culture transport glucose by a specific, saturable system with characteristics compatible with facilitated diffusion. Phagocytosis of killed staphylococci or polystyrene latex spheres results in a significant increase in uptake of 2-deoxy-D-glucose. Reciprocal plot analysis showed that the K_m values were lowered as a consequence of phagocytosis by a factor of between 2 and 3 in both cell types; V_{max} values were not significantly changed. The nature of the intracellular sugar pool was analyzed and found to consist of free and phosphorylated 2-deoxy-D-glucose at a relatively constant ratio of 1:2 after periods of uptake between 1 and 20 min. Phagocytosis resulted in increased levels of both free and phosphorylated sugars in the cytoplasm. Since the K_m values were lowered, augmented glucose uptake could not be accounted for by altered hexokinase activity. It was concluded that phagocytosis induces changes in the glucose transport system per se. The data are compatible with the metabolic changes known to be associated with particle ingestian by phagocytic cells. The mechanism by which glucose transport is augmented after loss of significant amounts of cell surface during the phagocytic process is not yet known.

c) Transport of glucose and amino acids after immunological activation of peritoneal macrophages. Immunologically activated peritoneal macrophages from inbred mice and Hartley strain guinea pigs demonstrate a markedly greater than normal transport of 2-deoxy-D-glucose and L-leucine. The degree of nutrilite transport enhancement was greatest when animals were injected with the appropriate eliciting antigens before

harvesting and also, if antigen was included in the tissue culture medium during the initial hours of in vitro culture. Enhanced hexose and amino acid uptake could also be achieved by exposure of macrophages from nonimmunized animals for 48 hrs to supernatants of sensitized splenic lymphocyte cultures incubated with specific antigens. The animal systems in which this phenomenon was observed included CBA/J and C57BL/6J mice immunized with Staphylococcus aureus or sub-lethal doses of Listeria monocytogenes, and the Hartley strain, albino guinea pig immunized with S. aureus or BCG. In all cases, immunization resulted in a state of delayed hypersensitivity as measured by skin testing or footpad swelling. Splenic cell supernatants contained lymphokines as detected by the presence of macrophage inhibitory factor (MIF), and by the supernatants' capacity to stimulate incorporation of ^{14}C-glucosamine by macrophages in vitro. No increase of glucose or leucine transport by macrophages was observed in the absence of appropriate antigen stimulation in vivo or in vitro.

We previously showed that a phagocytic stimulus results in a significant increase in hexose transport by normal macrophages; leucine transport by these same cells was unaltered after phagocytosis. In contrast, immunologically activated macrophages do not transport measurably more 2-deoxy-D-glucose after particle ingestion; activation or the phagocytic stimulus enhance 2-deoxy-D-glucose uptake to approximately the same extent. Analysis of nutrilite transport kinetics revealed that immunological activation of macrophages increases the initial velocity (V_i) and V_{max} but does not change the K_m values of hexose or amino acid transport. The kinetics of transport by the immunologically activated macrophages do not change measurably after phagocytosis. We conclude that either immunological activation or phagocytosis results in augmented 2-deoxyglucose transport via identical or related mechanisms and that transport of the sugar cannot be increased above that level induced by either event. The reasons why immunological activation increases both glucose and leucine transport but phagocytosis increases only the former are not yet understood.

d) Membrane transport by guinea pig macrophages and polymorphonuclear leukocytes. Short term, carrier mediated transport of D-glucose, L-leucine and L-lysine by guinea pig peritoneal macrophages was characterized. Analysis of the amino acid transport demonstrated two-limbed double reciprocal plots suggesting two transport systems for each amino acid. The low concentration limb of the curves established a K_m of 0.1 mM for L-leucine and 0.05 mM for L-lysine; Vm_{ax} values were 2.0 and 2.85 nmole/mg protein/90 sec, respectively. Leucine and lysine were shown to be competitive inhibitors of each other. Further competition studies revealed that other amino acids also had affinity for these carriers. Amino acid transport was found to be sensitive to sulfhydryl active compounds. Colchicine treatment of peritoneal macrophages did not inhibit the transport of the amino acids tested. Preloading macrophages with latex beads or heat-killed staphylococci by phagocytosis stimulated 2-deoxy-D-glucose (2-doG) uptake markedly, but had no measurable effect on amino acid transport. Although total transport of 2-doG increased in post-phagocytic macrophages, the kinetics of the system were not altered significantly. The K_m for both pre- and post-phagocytic transport of 2-doG was shown to be 1.2 mM and the V_{max} was shown to increase from a pre-phagocytic value of 20 nmoles/mg protein/90 sec to a post-phagocytic 27 nmoles/mg protein/90 sec. Phagocytosis of heat-killed staphylococci by guinea pig polymorphonuclear leukocytes (PMNs), however, did not cause an augmentation in hexose transport in the cells. The presence of colchicine during phagocytosis did not alter subsequent uptake of amino acids by the macrophages.

e) Mechanism of enhanced glucose transport by macrophages after phagocytosis. Short term carrier mediated transport of L-leucine, L-lysine and D-glucose in guinea pig macrophages and polymorphonuclear leukocytes (PMNs) was characterized. Carrier mediated transport of glucose in mouse macrophages was also examined. Analysis of amino acid transport in the guinea pig polymorphonuclear leukocyte demonstrated two-limbed double reciprocal plots suggesting two transport systems for each amino acid. The low concentration limb of the curves established a K_m of approximately 0.10 mM for both L-leucine and L-lysine; V_{max} values were found to be 0.8 and 1.8 nanomoles/mg protein/90 sec, respectively. Phagocytosis of heat-killed staphylococci by guinea pig PMNs did not alter their amino acid transport systems. Treatment of these cells with colchicine prior to phagocytosis in the presence of colchicine did not alter subsequent amino acid transport. The total transport of 2-deoxy-D-glucose (2-doG) increased about 35% in post-phagocytic mouse macrophages, as compared to normal phagocyres, and the kinetics of the mouse peritoneal macrophage system appeared to be similar to that measured in guinea pig macrophages. In guinea pig macrophages, the nature of the intracellular sugar pool was analyzed and was found to consist of free and phosphorylated 2-doG at a relatively constant ratio of 1:3 after periods of uptake between 15 and 90 sec, and 1:7 after uptake for 5 and 10 minutes uptake. Phagocytosis of heat-killed staphylococci by the guinea pig macrophage resulted in increased ratios of free to phosphorylated 2-doG of 1:6 after uptake periods of 90 sec, and 1:11 after uptake periods of 5 and 10 minutes. Whether the augmented glucose uptake reported herein is directly or indirectly affected by this altered hexokinase activity is not immediately known.

f) Phagocytic and bactericidal activity of mouse peritoneal macrophages. Although Staphylococcus aureus is incapable of intracellular multiplication in cultured mouse peritoneal macrophages, it is killed at a much slower rate than the avirulent Staphylococcus epidermidis. In addition to the presence of capsular material which inhibits phagocytosis of specific strains of S. aureus, the data show that a number of cellular and environmental factors affect the functional capacities of mononuclear phagocytic cells. The data obtained by varying the initial level of infection indicate that the number of ingested bacteria may subsequently alter the kinetics of intracellular killing. In vitro maturation of macrophages in culture was also found to exert a pronounced effect on the kinetics of bacterial death.

g) Cell mediated immunity induced in chronic staphylococcal infections. Splenic lymphocytes from normal mice and from mice displaying delayed hypersensitivity to Staphylococcus aureus were cultured in the presence or absence of specific staphylococcal antigens. The cell free supernatant fluids from these lymphocyte cultures were assessed for their ability to alter the functional capacities of normal macrophages. It was found that supernatants from staphylococcus-immune cells culture in vitro with antigen possessed migration inhibitory factor activity and also were capable of stimulating the incorporation of (^{14}C) glucosamine into macrophage membrane glycoproteins. In addition, the lymphokine-containing supernatants were capable of inducing activation of normal macrophages so that they inhibited the multiplication of intracellular Listeria monocytogenes. Although it was not possible to show any significant enhancement of intracellular killing of S. aureus by the activated macrophages, evidence is presented that suggests that cell-mediated immune responses to S. aureus may significantly enhance phagocytosis of staphylococci and, thereby, may provide for their rapid clearance from extracellular fluids.

h) Non-specific resistance induced by chronic staphylococcal infections. Normal mice and mice displaying delayed hypersensitivity to Staphylococcus aureus were challenged with a lethal dose of Listeria monocytogenes. Nonspecific antimicrobial activity was assessed by inhibition of bacterial growth in the spleen and by increased survival rates. Administration of specific staphylococcal antigen prior to challenge was a prerequisite for the induction of nonspecific resistance. Both the time of administering eliciting antigen and the route were important consideration for evoking the response. Similar responses were seen in 3 strains of inbred mice following immunization with both encapsulated and nonencapsulated staphylococci. Although enhanced resistance as measured by viable counts in the spleens was achieved after 2 injections, protection as measured by survival required 4 injections of S. aureus followed by elicitation with staphylococcal antigens. Nonspecific resistance could be detected as late as 9 weeks after the disappearance of delayed hypersensitivity.

i) Transfer of resistance by lymphocytes obtained from mice chronically infected with Staphylococcus aureus. Cell-mediated immunity following multiple staphylococcal infections of mice was found to differ from other established experimental models of infection with facultative intracellular microorganisms in that acquired cellular resistance was of extremely short duration. This is perhaps a reflection of the fact that Staphylococcus aureus does not multiply or survive within mononuclear phagocytes and are eliminated from the tissues within a few days. Thus, a sustained antigenic stimulus required for maintenance of cellular immunity does not occur. Spleen cells from immunized mice transferred simultaneously with staphylococcal antigen conferred resistance against Listeria monocytogenes on unimmunized syngeneic mice. Treatment of immune splenic lymphocytes with antilymphocyte serum and complement markedly inhibited or abolished capacity of the lymphocytes to transfer resistance to Listeria. These results support and extend our previous data which suggest that mice infected repeatedly with staphylococci are able to suppress the growth of L. monocytogenes via cellular rather than humoral mechanisms.

j) Interaction of diphtheria toxin with phagocytic cells. The interaction of the toxin of Corynebacterium diphtheriae with leukocytes from sensitive and resistant animal species was examined by determining the ability of toxin to inhibit protein synthesis by several types of phagocytic cell. Small amounts of toxin (25 minimal lethal doses) impaired protein synthesis in both polymorphonuclear leukocytes and mononuclear cells from humans and guinea pigs, whereas large amounts (2,000 minimal lethal doses) were required for minimal inhibition of mouse phagocytes. Peritoneal macrophages from hyperimmunized guinea pigs exhibited the same high degree of sensitivity to diphtheria toxin as did those from unimmunized animals. Prolonged incubation with toxin resulted in a 75% reduction in phagocytosis of polystyrene latex spheres but had no effect on transport of the glucose analogue 2-deoxy-D-glucose by guinea pig macrophages. Thus phagocytic cells, although they are endowed with a high level of endocytic activity, respond to diphtheria toxin as do somatic cells. All types of phagocytic cell appear to reflect the native resistance or sensitivity of the host species of origin.

k) Uptake of diphtheria toxin and fragment A by non-professional phagocytes. Evidence suggesting that diphtheria toxin reaches the cytoplasm of susceptible mammalian cells by two independent mechanisms is presented. A schematic model describing the two processes of toxin entry into the cell is developed. One process of toxin uptake considered to be physiologcally significant is passage of the protein toxin through the plasma membrane.

This most likely happens by binding of fragment B to receptors on the membrane and by subsequent toxin-membrane interaction so that ultimately fragment A, the enzymatically active moiety, is transported to the cell interior. This process, which ultimately leads to cessation of protein synthesis and cell death, involves a comparatively small number of toxin molecules. A second mechanism of toxin uptake is by classical pinocytosis. The majority of toxin taken into the cell is accomplished by this process. The fate of toxin taken into HEp-2 cells via pinocytoxis is proteolysis by lysosomal enzymes. Thus, such vesicle-bound toxin is ordinarily not expressed biologically. Evidence suggesting tha ammonium chloride provides total protection to diphtheria toxin-susceptible cells by preventing entry of toxin by the specific receptor-associated process is also provided; data showing that the ammonium salt immobilizes bound toxin on the plasma membrane of HEp-2 cells are presented. Finally, it is suggested that actively endocytic cells such as guinea pig macrophages interact with toxin in a significantly different manner than do nonphagocytic cells.

l) Activated macrophages in athymic (nude) mice. The discovery of the congenitally athymic nude mouse in 1966 presented a valuable model for the study of bacterial infections in the absence of T-lymphocyte regulated immune mechanisms. The nude mutation affords a model unaffected by any extraneous effects induced by radiation or antithymocyte serum treatment formerly used to deplete the T-lymphocyte population.

The majority of the experiments in this study utilized Listeria monocytogenes as the infectious agent and nude mice possessing a Balb/c genetic background. This organism was of particular interest since it has been conclusively demonstrated that L. monocytogenes is a facultative intracellular parasite capable of survival and multiplication within normal, nonactivated host macrophages. Protection against organisms of this type requires the generation of a population of T-lymphocytes specifically sensitized to microbial antigens. Products of the sensitized lymphocytes (lymphokines) induce activation of macrophages which in turn demonstrate an augmented bactericidal capacity; this cell-cell cooperation mediates elimination of the infectious agent from the tissues. Initial studies demonstrated an anomalous resistance in the nude mouse in spite of the T-cell deficit. Nude mice were more resistant to L. monocytogenes infection than immunocompetent (heterozygote) littermates. Levels of Listeria challenge (i.v.) sufficient to kill immunocompetent animals were not lethal for nude mice. However, the athymic mice were unable to completely eliminate the infecting organisms from tissues and subsequently developed a chronic infection. Nude mice also developed a chronic low-level infection after administration of a challenge dose of L. monocytogenes sublethal for heterozygote mice; the latter eliminated listeria completely after development of specific cell-mediated immunity. The absence of T-cells precluded development of effective cell-mediated immunity by the nudes.

Additional in vivo studies were conducted to determine the nature of the high resistance to infection of the nude and to determine the cellular and/or humoral mechanisms responsible. The high natural resistance, determined by rapid blood clearance and low viable bacterial counts in the reticuloendothelial organs, was demonstrable as early as three hours after intravenous challenge with L. monocytogenes, Staphylococccus aureus or Salmonella typhimurium. The early expression and nonspecific nature of the paradoxical resistance indicated the presence of activated tissue macrophages in the nude

mice prior to bacterial challenge. This inference was supported by additional experiments demonstrating a greater bactericidal capacity during the early hours of infection of the reticuloendothelial organs of the nude mice, as compared with heterozygotes. It was determined that the cell(s) responsible for resistance are derived from bone marrow. In vitro assays and a combined in vivo-in vitro technique suggested that, in contrast to fixed tissue macrophages, peritoneal macrophages of nude mice were not activated as measured by phagocytic or bactericidal capacities. Nude mice which had received thymus grafts lost their heightened resistance to infection within 5 weeks of implantation. This observation indicates that activation of fixed tissue macrophages is intimately associated with T-cell deficiency.

Evidence is also presented suggesting that the bacterial and viral flora of conventionally reared nude mice contributes significantly to the activated state of tissue macrophages. The immunological basis for the anomalous resistance of athymic nude mice remains to be established conclusively.

m) Interaction of normal and malignant cells with diphtheria toxin. Malignant transformation of rodent cells does not alter their response to the cytotoxic action of diphtheria toxin (DT). Transformed cells of mouse and rat origin are of comparable resistance as normal cells from these toxin resistant species judged by an in vitro assay employing monolayer cultures. The TC_{50} dose for inhibition of protein synthesis in a variety of normal and malignant rodent cell lines is greater than 3.0 $\log_{10}$ units higher than that for cultures derived from human tissues. These comparative values are consistant with the relative toxin resistance or susceptibility of the respective animal species of cell origin. It is concluded that the malignant status of mammalian cells has no meaningful bearing on the susceptibility of that cell to the cytotoxic action of diphtheria toxin. Determinants of toxin sensitivity or resistance apparently reside at the level of the plasma membrane but are unrelated to surface properties associated with malignant cells.

5. BIBLIOGRAPHY

For publications see 3B. In addition to these 8 publications there are:
2 papers are in press
2 papers have been submitted for publication
1 manuscript is in preparation.
These 5 manuscripts are listed below.

Enhancement of carrier mediated transport after immunological activation of peritoneal macrophages. P. F. Bonventre, D. Straus, R. E. Baughn, and J. Imhoff. 1977. J. Immunol. (in press).

Normal and malignant rodent cells demonstrate comparable resistance to diphtheria toxin. C. B. Saelinger and P. F. Bonventre. 1977. J. Nat. Canc. Inst. (in press).

Membrane transport of amino acids and hexose by guinea pig and mouse phagocytes. D. C. Straus, J. Imhoff and P. F. Bonventre. Submitted for publication to J. Reticuloendothelial Society.

Membrane transport by guinea pig peritoneal exudate leukocytes: Effect of phagocytosis on hexose and amino acid transport. D. C. Straus, J. Imhoff and P. F. Bonventre. Submitted for publication in the J. Cell. Physiology.

Anomalous resistance of congenitally athymic mice to bacterial infections. A. Nickol and P. F. Bonventre (for submission to Infection and Immunity).

6. APPENDIXES

Copies of reprints as listed in 3 b are appended.

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Copies of reprints as listed in 3 b are appended.